TROPICAL AND GEOGRAPHICAL MEDICINE 1987, Vol 39 n°3 ; 281-286

SPECIFIC IMMUNODIAGNOSIS OF CHAGAS DISEASE: IMMUNODIFFUSION TEST USING A SPECIFIC SERUM ANTI-TRYPANOSOMA CRUZI COMPONENT 5

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Received December 12, 1985
Accepted for publication May 5, 1987

Abstract A micro double diffusion test (MD), allowing the identification of precipitation band 5 by identity reaction, using a rabbit specific anti-component 5 serum, was evaluated for the immunological diagnosis of Chagas' disease. The previous studies on the *Trypanosoma cruzi* specificity of component 5[g] were completed, showing it to be absent in *Leishmania brazilienis*, but present in different strains of *T. cruzi*. 200 sera from Bolivian patients were studied. (88 with a positive xenodiagnosis, 45 with mucocutaneous leishmaniasis but without Chagas' disease, and 67 controls). Band 5 was found in 74 (84.1%) of the sera with positive xenodiagnosis but was never found either in the leishmaniasis or in the control groups. MD, allowing an easy detection of *T. cruzi* specific band 5, cheap and simple to perform, can be recommended in association with other serological tests, when highly specific immunodiagnosis of Chagas' disease is required

Key words: specific immunodiagnosis, Chagas' disease, Trypanosoma cruzi component 5, immunodiffusion; Bolivia

Introduction

The importance of the immunodiagnosis of chronic Chagas' disease has already been widely emphasized. However, many human sera with positive serological reactions for *Trypanosoma cruzi* also have positive reactions with antigens of other flagellates such as *T. rangeli, Leishmania donovani* or *L. braziliensis* [1-8]. The existence of shared epitopes between flagellate antigens [9-11] can explain such observations. Consequently various methods have been proposed to increase the specificity of the immunodiagnosis of Chagas' disease, using: 1. other parasite forms, like amastigotes or trypomastigotes, assumed to be more specific for *T. cruzi* than epimastigote forms [5, 12, 13]; 2. other antigenic extraction procedures [7]; 3. absorption of the positive human sera with the cross-reacting antigens, before determination of the anti-*T. cruzi* antibody level [3]; 4. specific purified antigen of *T. cruzi* [14, 15].

Such a *T. cruzi* antigen, the so-called component 5, has been demonstrated without shared epitopes with *L. donovani, L. mexicana, T. brucei* or *T. rangeli* [9, 16]. Moreover, using immunoelectrophoresis (IEP), anti-component 5 precipitating antibodies could be demonstrated in 72.6% of sera from patients chronically infected by *T. cruzi* [17]. The aims of this work was to complete the specificity study of component 5 and to evaluate a micro double diffusion test (MD), allowing the identification of precipitation band 5 by identity reaction using a specific rabbit anti-component 5 serum, for specific immunodiagnosis of Chagas' disease. MD, previously used for specific diagnosis of other parasitic disease [18], was expected to be simple to perform, avoiding preparation of a large quantity of purified component 5, and higher sensitive than IEP.

Materials and Methods

Human sera.
Sera were obtained from 200 Bolivian patients, divided into three groups according to their geographical origin. The first group (1) contained 88 patients (mean age 39 ± 13 years) with positive xenodiagnosis. They came from southern lowlands, areas known to be highly endemic for Chagas' disease, but where leishmaniasis had never been found. They were asymptomatic or with cardiac or digestive symptoms compatible with the chronic phase of Chagas' disease. The second group (2) contained 45 patients (mean age 29 ± 9 years) with clinical evidence of mucocutaneous leishmaniasis (35 with typical primary cutaneous ulcerations with surrounding indurations and 10 with typical mutilations of the face). They lived in northern lowlands ('Beni' and 'Alto Beni'), areas known to be endemic for leishmaniasis but free of Chagas' disease. The third group (3) was a control group of 67 asymptomatic patients (mean age 25 ± 5 years), from highland areas ('Altiplano') exempt of both infections and who had never travelled in the endemic areas.

T. cruzi and L. braziliensis antigens.
T. cruzi epimastigotes (Tehuantepec strain) were cultivated in cell free GLSH monophasic medium at 28°C [19]. After 7 culture days, the parasites were harvested by centrifugation at 800 g for 15 min at 4°C and washed three times with Hank's balanced salt solution. Six grams (wet weight) of epimastigotes were suspended in 36 ml of 1% NaCl, frozen and desintegrated five times in a hydraulic press (LKB X press) at 18000 psi and then centrifuged at 26.000 g for 1h at 4°C. The supernatant was dialyzed against distilled water for 24h at 4°C, lyophilized and used as a soluble extract of *T. cruzi*. Soluble antigenic extracts of other six different isoenzymic strains of *T. cruzi*, classified as 1, 1b, 1c, 2, 2a and 2b according to Tibayrenc *et al.* [20] were prepared as the *T. cruzi* Tehuantepec strain for the complementary study of specificity. *L. braziliensis* promastigotes (LV65 strain) were obtained from cell-free culture on NNN medium, modified according to Deker-Jackson & Honigberg [21]. A. *L. braziliensis* soluble extract was prepared as for *T. cruzi*.

T. cruzi component 5. 20 mg of *T. cruzi* (Tehuantepec strain) soluble antigenic extract were resuspended in 4 ml of distilled water and added with an equal volume of chloroform/methanol solution (2/1). The mixture was shaken for 30 min at room temperature and centrifuged at 1.000 g for 30 min at 4°C. The aqueous phase was collected and extracted twice in the same way. Organic solvents were evaporated and glycoproteins were precipitated by the addition of 3 volumes of ethanol for 4 hours at -20°C. After centrifugation (1.000 g - 30 min), the precipitate was washed with ethanol, dried, and resuspended in 2 ml of distilled water. The solution was centrifuged, dialyzed against distilled water for 24 hours at 4°C and lyophilized to obtain 2.5 mg of a component 5-rich fraction.

Rabbit immune sera.
One rabbit was immunized with the total soluble extract of *T. cruzi*, for the specificity study of component 5. Another rabbit was injected with fraction 5 and used in the diagnosis evaluation of MD. The immunization procedure used simultaneous multiple intradermic injections, according to Vaitukaitis *et al.* [22] with 2 mg of antigen. The rabbits were boosted by weekly subscapular injections of 1 mg of antigen over six weeks. The presence of precipitating anti-component 5 antibodies in the rabbit sera was controlled in IEP and in immunodiffusion by identity reaction with a reference monospecific anti-component 5 serum, prepared according to Afchain *et al.* [9].

Micro double diffusion test (MD).
MD was performed on microscope slides (25 × 80 mm) covered with 4 ml of 1% agarose (IBF-France) in 0.1 M veronal buffer, pH 8.2. Three patterns of wells for sera and antigenic extract were punched for one slide, allowing simultaneous study of 12 different human sera (*figure 1*). 60 μl of human sera, concentrated to 12 μl by lyophilization, was placed in peripheral wells whereas 12 μl of 1/4 diluted rabbit anti-5 immune serum was put in the central wells. The two micro-wells were filled with 2 μ (24 μg) of *T. cruzi* antigenic extract.

Then, the slides were incubated in a moist chamber, at room temperature for 24h. They were washed by immersion in 0.1 M veronal buffer pH 8.2 for 48h, demineralized in distilled water for 1h at room temperature, dried and stained by Coomassie blue. The test was positive when one of the precipitation bands produced by the human sera showed an identity reaction with the band 5 obtained with the anti-component 5 immune serum.

Results

Complementary study on the T. cruzi specificity of component 5. As shown in *figure 2*, precipitation band 5 was demonstrated to be present and identical in the 6 studied different isoenzymic strains of *T. cruzi*, but it was not found in the *L. braziliensis* antigenic extract.

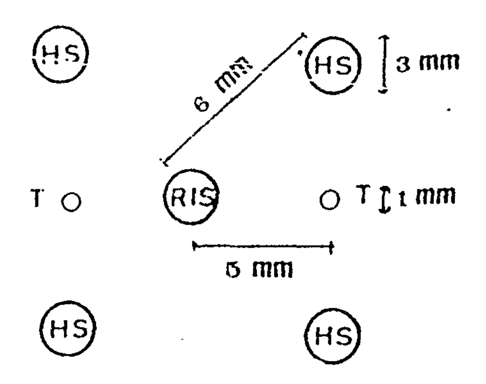


Figure 1. MD pattern (RIS = anti-component 5 rabbit immune serum; HS = human serum; T = soluble antigenic extract of *T. cruzi*).

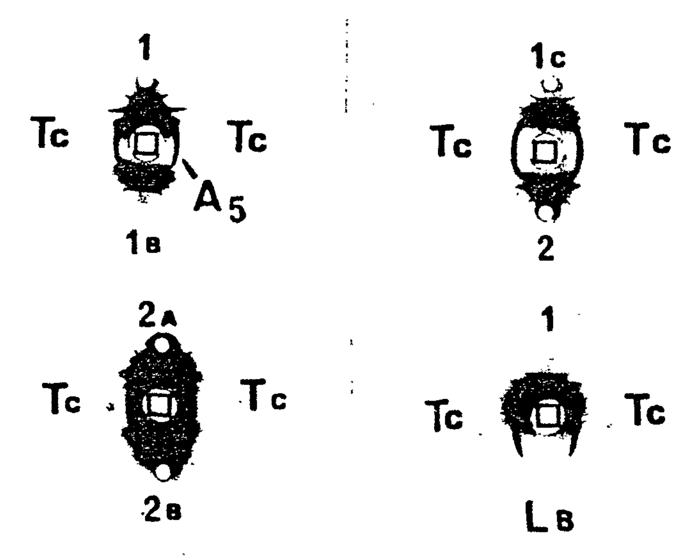


Figure 2. Immunodiffusion test studying the specificity of component 5 (1, 1B, 2, 2A, 2B = soluble antigenic extract of different isoenzymic strains of *T. cruzi* containing component 5 (A5); Tc = component 5 fraction (*T. cruzi* Tehuantepec strain; LB = soluble antigenic extract of *L. braziliensis* □ = anti *T. cruzi* rabbit serum).

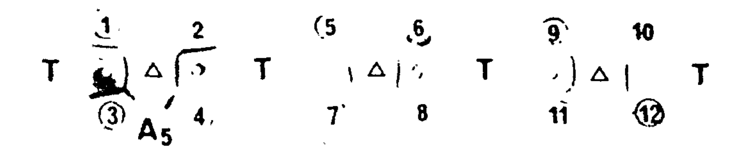


Figure 3. MD with human sera (1, 2, 3, 9, 10, 11: human sera showing precipitation band 5 (A5); 4, 5, 6, 7, 8, 12 = band 5 negative sera; T = *T. cruzi* antigenic extract △ = anti-component 5 rabbit immune serum).

Evaluation of the micro double diffusion test to identify the precipitation band 5. 74 out of 88 (84,1%) sera from the group 1 presented a well-defined precipitation band, identical to the band obtained with the specific anti-component 5 sera in MD, as shown in *figure 3*. No sera of leishmaniasis group 2 and of control group 3 presented a precipitation band.

Discussion

The *T. cruzi* specificity of component 5 in relation to *T. brucei*, *T. rangeli*, *L. donovani*, and *L. mexicana* was previously demonstrated by Afchain *et al.* [9, 16]. The present results completed this specificity study, showing its presence in different *T. cruzi* strains but the absence of component 5 in *L. braziliensis*. Moreover band 5 was not found in sera from patients with mucocutaneous leishmaniasis or Bolivian controls. Consequently it can be affirmed that the presence of anti-component 5 antibodies in a serum ensures the diagnosis of Chagas' disease.

The MD sensitivity of band 5 detection was 84.1%, which is higher than the 72.6% previously obtained using IEP [17]. Indeed MD avoids the electrophoresis step, necessary to identify component 5 in IEP, increasing the antigen concentration available for the diffusion step. Moreover, in MD, the identification of band 5 in human serum is easy using the identity reaction with a rabbit specific anti-component 5 immune serum.

MD requires little human or rabbit serum or *T. cruzi* antigenic extract and allows simultaneous study of many sera using microscope slides. The specific anti-component 5 serum is easy to produce. 40 ml of a high quality serum, from one immunized rabbit, allow 40.000 patient sera to be tested. Moreover human sera with a predominant precipitation band 5 could be progressively selected by IEP or immunodiffusion to be used instead of rabbit serum.

Since other serological tests, such as immunofluorescence, complement fixation test or ELISA, also with Bolivian sera, though less specific, obtained a better relative sensitivity (99-100%) [17], the routine use of MD for the immunodiagnosis of Chagas' disease, instead of more sensitive tests, is unsuitable. However, when the classical serological tests are positive for different flagellate antigens, this can be due to shared epitopes between flagellates or mixed infections with various flagellates. Indeed, most South american areas are known to be endemic for both leishmaniasis and Chagas' disease and it is essential to obtain a confirmation of the diagnosis of Chagas' disease, since clinical and therapeutic management of leishmaniasis and Chagas' disease are very different. In such cases, in association with other serological tests, MD, with identity reaction, cheap and simple to perform (not requiring enzyme or fluorochrome-labelled reagents), can be recommended as a highly specific tool for immunodiagnosis of Chagas' disease.

We are grateful to Dr. M. Tibayrenc for typing the *T. cruzi* strains and for the diligent technical assistance of Hortensia Miguez, Clara Camacho, Ana Maria Monjon and Olivier Poch.
This study has been supported by the French Ministry of Foreign Affairs and by the Ministry of Research and Industry (Grant n° PVD/81-L-1423); Belgian FNRS (Grant n° 1.5.603.83F) and CST (18.114 bis), EEC (Grant n° TSD-M 024B) (RS) and the WHO (Special Programme for Research and Training in Tropical Diseases).

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